LEPTOSPIRA PATHOGENS

The present invention relates generally to novel isolated species of pathogenic bacteria and to immunoreactive molecules which are derived therefrom and their use in compositions of matter such as vaccine preparations. The compositions of the present invention are useful in protecting host organisms against bacterial infections. More particularly, the present invention is directed to an isolated serogroup, serovar or species of bacteria belonging to the genus *Leptospira*. Even more particularly, the present invention is directed to a new isolated species of *Leptospira* designated as "*L. fainei* or a new isolated *L. fainei* serovar designated as "hurstbridge" and to bacteria belonging to the same serogroup as serovar hurstbridge or *L. fainei*, designated as "serogroup Hurstbridge" and to diagnostic assays therefor. The present invention is further directed to methods of detection, identification and quantification of *Leptospira*, such as those *Leptospira* belonging to serogroup Hurstbridge and more particularly to methods of detection of *L. fainei* and even more particularly to methods of detection of serovar hurstbridge. The present invention further provides vaccine compositions which provide for the passive and active vaccination of human or animal hosts against *Leptospira*, such as those *Leptospira* belonging to serogroup Hurstbridge and more particularly to vaccine compositions against *L. fainei* serovar hurstbridge.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

Bibliographic details of the publications referred to by author in this specification are

collected at the end of the description. Sequence identity numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined after the bibliography.

Bacteria of the genus *Leptospira* are either pathogenic or saprophytic spirochaetes comprising several known species (Pathogenic: *L.interrogans*, *L.inadai*, *L.borgpetersenii*, *L. santarosai*, *L. kirschneri*, *L. weilii* or *L. noguchii* ; Saprophytic: *L.biflexa*, *L. meyeri* or *L. wolbachii*), each of which comprises a large number of serovars. Saprophytic serovars of *Leptospira* are omnipresent in fresh surface waters and occasionally found in sea water. Pathogenic *Leptospira* serovars occur naturally in a large variety of livestock animals, companion animals, wild animals and humans. The host range of *Leptospira* serovars is generally quite broad, however the bacterium may produce differing symptoms in each host organism which it has infected.

In a primary (maintenance) host in which a pathogenic *Leptospira* serovar is maintained, reproductive disease is typical. Alternatively, infection may be asymptomatic. Pathogenic *Leptospira* serovars may also cause acute, febrile, systemic disease in mammals. Acute febrile disease is also characteristic of many human infections.

In livestock animals such as pigs and possibly horses and dogs or other species, the pathogen *L.interrogans* serovar bratislava causes reproductive disease leading to infertility, abortions or stillbirth and has been cited as a possible causative agent of seasonal infertility (Chappel *et al.*, 1993a,b; Ellis *et al.*, 1983; Ellis *et al.*, 1985; Ellis *et al.*, 1986a,b; Frantz *et al.*, 1988). Infection with *L.interrogans* serovar bratislava is endemic in European and North American swine herds. In Australian swine herds, the pathogenic serovars *L.interrogans* serovar pomona and *L. borgpetersenii* serovar tarassovi have long been recognised (Chappel *et al.*, 1987a,b; Chappel *et al.*, 1990; Davos, 1977), however many Australian herds have also tested positive for the presence of *L.interrogans* serovar bratislava using the microscopic agglutination test, hereinafter referred to as "MAT" (Chappel *et al.*, 1992; Chappel *et al.*, 1993a,b). *Leptospira interrogans* serovar bratislava

is notoriously recalcitrant to standard isolation techniques, using samples from the infected host organism as starting material. This factor has to date prevented the preparation in Australia of vaccines which protect animals specifically against infection by serovar bratislava.

In work leading up to the present invention, the inventors sought to isolate *Leptospira* serogroups and serovars, in particular *L.interrogans* serovar bratislava from swine herds with MAT titres to serovar bratislava and with immunochemical evidence of leptospiral infection. Surprisingly, a novel leptospire was isolated which does not cross-react in MATs with other pathogenic serovars including serovars bratislava, pomona and tarassovi. This new leptospire forms an antigenically-distinct serogroup and serovar, based upon microscopic agglutination assay (MAT) results and a genetically-distinct species, based upon nucleic acid hybridisation data. The new *Leptospira* and recombinant nucleic acid, polypeptides or immunoreactive molecules which are derived therefrom, and derivatives, homologues or analogues thereof, provide the means to develop a range of diagnostic and therapeutic agents for *Leptospira* infection which were hitherto not available.

Accordingly, one aspect of the present invention provides an isolated pathogenic *Leptospira* bacterium which belongs to serogroup Hurstbridge or serovar hurstbridge or the species *L. fainei* or derivative bacterium thereof.

The terms "serogroup" and "serovar" relate to a classification of Leptospira which is based upon serological typing data, in particular data obtained using agglutination assays such as the microscopic agglutination test (MAT).

The term "serovar" means one or more *Leptospira* strains which are antigenically-identical. Quantitatively, serovars are differentiated from one another by the cross-agglutination absorption technique as outlined by Faine (1994).

In the present context, the term "serovar hurstbridge" shall be taken to include any leptospire which is cross-reactive according to the cross-agglutination absorption criteria (Faine, 1994) with the *Leptospira fainei* strain WKID deposited under AGAL Accession No. N95/69684 or the *L. fainei* strain BUT6. The term "serovar hurstbridge" is not to be limited in any way to those bacteria belonging to serogroup Hurstbridge as defined herein.

The term "serogroup" refers to a group of *Leptospira* whose members cross-agglutinate with shared group antigens and do not cross-agglutinate with the members of other groups and, as a consequence, the members of a serogroup have more or less close antigenic relations with one another by simple cross-agglutination.

Accordingly, the term "serogroup Hurstbridge" refers to a serological group of *Leptospira* whose members cross-agglutinate with shared group antigens of the *Leptospira fainei* strain WKID deposited under AGAL Accession No. N95/69684 or the *L. fainei* strain BUT6, however do not cross-agglutinate in a simple cross-agglutination test with the members of other groups known to those skilled in the art at the date of the present invention. The term "serogroup Hurstbridge" is not to be limited in any way to those bacteria belonging to serovar hurstbridge as hereinbefore defined.

The classification of *Leptospira* into different species will be known to those skilled in the art to refer to one leptospire whose total genomic DNA is less than 40% homologous to the genomic DNA of another leptospire. Accordingly, as used herein, the species definition "*Leptospira fainei*" or "*L. fainei*" shall be taken to refer to any leptospire bacterium which comprises genomic DNA which is at least 40% homologous to the genomic DNA derived from the *Leptospira* deposited under AGAL Accession No. N95/69684 or the *Leptospira* strain BUT6, as determined using standard genomic DNA hybridisation and analysis

techniques.

Those skilled in the art will be aware that serovar and serogroup antigens are a mosaic on the cell surface and, as a consequence there will be no strict delineation between bacteria belonging to serovar hurstbridge and/or serogroup Hurstbridge. Moreover, leptospires which belong to different species may be classified into the same serovar or serogroup because they are indistinguishable by antigenic determination.

The present invention clearly extends to any bacterium belonging to serovar hurstbridge or serogroup Hurstbridge or *Leptospira fainei*.

In connection with this invention, an exemplary *Leptospira fainei* bacterium of serogroup Hurstbridge or serovar hurstbridge has been deposited as depositor's reference WKID (VIAS), pursuant to and in satisfaction of, the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, with the Australian Government Analytical Laboratories (AGAL), 1 Suakin Street, Pymble, New South Wales 2073, Australia (Postal Address: PO Box 385 Pymble NSW 2073 Australia) on 15 November, 1995 and accorded AGAL Accession Number N95/69684.

The *Leptospira* strains WKID and BUT6 were originally isolated from different herds of pigs in New South Wales, Australia and Victoria, Australia as described in the Examples (*see*, for example Table 3). Both of these isolates belong to the species now known as *Leptospira fainei*, based upon DNA hybridisation analysis, as well as belonging to the serogroup Hurstbridge and serovar hurstbridge, based upon serological criteria using MAT.

A "derivative" of the leptospiral bacterium of the invention is a bacterium which has been developed by mutation, recombination, conjugation or transformation of leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* as hereinbefore defined. Preferably, a derivative of serogroup Hurstbridge or serovar hurstbridge or *L. fainei* is serologically cross-reactive or immunologically cross-reactive with serogroup Hurstbridge or serovar

hurstbridge as defined herein or genetically-related to *L. fainei* as hereinbefore defined, in particular the leptospire assigned AGAL Accession Number N95/69684 or *Leptospira* strain BUT 6. It will be known to a person skilled in the art how to produce such derivatives.

Accordingly, this aspect of the present invention relates to isolated pathogenic *Leptospira* bacteria which are antigenically cross-reactive in MAT with one or more antigenic determinants of the *Leptospira* deposited under AGAL Accession No. N95/69684 or the *Leptospira* strain BUT6 exemplified herein and/or which comprise genomic DNA which is at least 40% homologous to the genomic DNA derived from the *Leptospira* deposited under AGAL Accession No. N95/69684 or the *Leptospira* strain BUT6 or a derivative bacterium thereof.

In a particularly preferred embodiment of the invention, the bacterium belonging to *L. fainei* or serovar hurstbridge or serogroup Hurstbridge grows at temperatures from about 13°C to about 37°C, preferably at 13°C to 37°C and more preferably at temperatures of about 13°C. Additionally, it is particularly preferred that the subject *Leptospira* grows in the presence of 8-azaguanine or 5-fluorouracil, more preferably at least 100µg/ml 8-azaguanine, even more preferably at least 150µg/ml 8-azaguanine, still even more preferably at least 200µg/ml 8-azaguanine and even still more preferably at least 250µg/ml. or up to and including a concentration of 500µg/ml 8-azaguanine.

In a more preferred embodiment, said bacterium is further capable of infecting a human or a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, bovine, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

In an even more preferred embodiment, said pathogenic *Leptospira* bacterium is capable of infecting said human or animal and inducing reproductive disease therein.

The term "reproductive disease" as used herein shall be taken to refer to any abnormality of the reproductive system of a human or other animal, in particular pigs or bovines which reduces the fecundity of said human or animal, for example an abnormality characterised by infertility of said human or animal including seasonal infertility or abnormal development of a foetus in said human or animal or spontaneous abortion of a foetus in said human or animal or failure to conceive by said human or animal. In the context of the present invention, the term "reproductive disease" shall also be taken to include reduced or slowed development, such as an increase in the weaning-to-mating period in animals which are infected during gestation or before becoming pregnant. Such reproductive disease is caused by infection of a human or animal with a pathogenic bacterium of the genus *Leptospira*, in particular leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof.

In an alternative embodiment, the present invention provides an isolated *Leptospira* bacterium or derivative bacterium thereof which contains genetic sequences from nucleotide of the 16S ribosomal RNA (rRNA) gene which are at least 85% identical to the rRNA genetic sequences of *Leptospira inadai* serovar lyme and less than 80% identical to the rRNA genetic sequences of *Leptospira biflexa* serovar patoc, wherein said pathogenic bacterium is capable of growing at temperatures from about 13°C to about 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a preferred embodiment, the *Leptospira* bacterium or derivative serovar of the present invention is further characterised as a pathogenic bacterium.

More preferably, the pathogenic bacterium of the invention is further capable of infecting a livestock human or animal, in particular a human or livestock animal selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas or a companion animal such as a dog or cat, amongst others.

According to this embodiment of the invention, wherein a pathogenic *Leptospira* bacterium infects said livestock animal, it is most preferred that said bacterium induces reproductive disease therein.

In another alternative embodiment of the present invention, there is provided an isolated *Leptospira* bacterium or derivative thereof which contains genetic sequences which are at least 80% identical to the nucleotide sequence set forth in any one of SEQ ID NOs:1-2 or 4-7 or a complement, or a derivative, homologue or analogue thereof.

It is preferred that the percentage identity to the nucleotide sequence set forth in any one of SEQ ID NOs:1-2 or 4-7 is at least 85%. According to this embodiment of the invention, it is more preferred that the genetic material of said pathogenic *Leptospira* bacterium or derivative bacterium thereof be at least 90% identical to any one of SEQ ID NOs:1-2 or 6-7, even more preferably at least 97% identical and still more preferably at least 99% identical including 100% identical.

For the purposes of nomenclature, the nucleotide sequence set forth in SEQ ID NO:1 relates to the nucleotide sequence of the rRNA gene of an isolate of serovar hurstbridge or serogroup Hurstbridge or *L. fainei*. The nucleotide sequences set forth in SEQ ID NOs:2-7 relate to primer sequences specific for the rRNA gene of serovar hurstbridge or serogroup Hurstbridge or *L. fainei* which the inventors have shown are particularly useful for the diagnostic detection of the pathogenic *Leptospira* bacterium of the species or serogroup Hurstbridge. More particularly, the nucleotide sequences set forth in SEQ ID NOs:2-3 are useful as a primer pair for the diagnostic detection of the pathogenic *Leptospira* bacterium of the species or serogroup Hurstbridge using the polymerase chain reaction.

In a further alternative embodiment, the present invention provides an isolated pathogenic *Leptospira* bacterium or derivative bacterium thereof which contains genetic material capable of hybridising under high stringency conditions to the nucleotide sequence set forth in any one of SEQ ID NOs:1-2 or 4-7 or its complementary nucleotide sequence, or a

derivative, homologue or analogue thereof.

Preferably, said genetic material is selected from the list comprising RNA or DNA.

In a further alternative embodiment, the present invention provides an isolated *Leptospira* bacterium or derivative bacterium thereof which contains a rRNA gene which comprises a nucleotide sequence which is at least 80% identical to the nucleotide sequence 5'-TGTTGGA-3' (SEQ ID NO:4) or at least 90% identical to the nucleotide sequence 5'-TTTGATA-3' (SEQ ID NO:5) or at least 90% identical to the nucleotide sequence 5'-TGTTGGA(N)$_8$TTTGATA-3' (SEQ ID NO:6) or a complement, or a derivative, homologue or analogue thereof, wherein N is any nucleotide residue.

More preferably, the isolated *Leptospira* bacterium of the present invention or a derivative bacterium thereof contains a rRNA gene which comprises a nucleotide sequence which is at least 85% identical to the nucleotide sequence 5'-TGTTGGATCACAAGATTTGATA-3' (SEQ ID NO:7) or a complement, or a derivative, homologue or analogue thereof.

According to this embodiment, the inventors have discovered a region of the rRNA gene of a leptospire which is unique to the species *L. fainei* or serogroup Hurstbridge or serovar hurstbridge belongs and is particularly suited for diagnostic applications. The present invention clearly extends to isolated nucleotide sequences and oligonucleotides which comprise said nucleotide sequences.

It is preferred that said pathogenic *Leptospira* bacterium is further capable of growing at temperatures from about 13°C to about 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

More preferably, the present invention provides an isolated pathogenic *Leptospira*

bacterium or derivative bacterium thereof which contains RNA or DNA capable of hybridising under high stringency conditions to the nucleotide sequence set forth in any one of SEQ ID NOs:1-2 or 4-7 or a complementary nucleotide sequence thereto or a derivative, homologue or analogue thereof, wherein said bacterium is further capable of growing at temperatures from about 13°C to about 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

For the purposes of defining the level of stringency, a high stringency is defined herein as being a Southern hybridisation and/or a wash thereafter carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at 65°C . In Southern hybridisations, the stringency is generally increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Conditions for Southern hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification, (to parameters affecting hybridisation between nucleic acid molecules), reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

Alternatively, a high stringency is also defined according to the conditions which are appropriate for the annealing of nucleic acid primers in a polymerase chain reaction (PCR) as exemplified herein.

In a particularly preferred embodiment of the present invention, there is provided an isolated bacterium or serogroup which:

1. Is a pathogenic species belonging to the genus *Leptospira*;
2. Grows at temperatures in the range from about 13°C to about 37°C;
3. Grows in media containing at least 225µg/ml 8-azaguanine;
4. Is capable of infecting a human or a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs,

bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others;

5. Is capable of inducing reproductive disease as hereinbefore defined in at least one of said infected animal; and

6. Contains a genetic sequence which comprises a sequence of nucleotides or is complementary to a genetic sequence which comprises a sequence of nucleotides which is at least 80% identical to the nucleotide sequence set forth in any one of SEQ ID NOs:1-2 or 4-7 or a complementary nucleotide sequence thereto or a derivative, homologue or analogue thereof.

In a most particularly preferred embodiment, the present invention provides an isolated pathogenic *Leptospira* bacterium belonging to serogroup Hurstbridge or serovar hurstbridge or *L. fainei* which possesses the characteristics or attributes of the microorganism deposited with AGAL under AGAL Accession Number N95/69684 or is within the same serogroup (as defined by Faine, 1994) as the microorganism N95/69684 or is in the same species as the microorganism N95/69684 or is immunologically cross-reactive with the microorganism N95/69684 in a microscopic agglutination test (MAT).

Even more preferably, bacteria belonging to serovar hurstbridge or serogroup Hurstbridge or *L. fainei* as defined herein are pathogens of humans and/or livestock or companion animals, in particular livestock or companion animals selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

A further embodiment of the present invention provides an isolated serovar of leptospiral serovar hurstbridge as hereinbefore defined or a derivative bacterium thereof. Preferably, said serovar of a leptospiral serovar hurstbridge is genetically-cross-reactive or immunologically-cross-reactive with the strain deposited with AGAL under Accession No. N95/69684 on 15 November, 1995.

More preferably, said serovar is identical to the strain deposited with AGAL under Accession No. N95/69684 on 15 November, 1995.

According to this embodiment of the invention, said isolated serovar may be determined to be immunologically-cross-reactive or genetically-cross-reactive or genetically-cross-hybridising with the serovar of the leptospire deposited under AGAL Accession No, N95/69684 by any means known to those skilled in the relevant art, including, but not limited to, serological, immunological, or molecular-biological means. Serological means include MAT titre estimations (Cole et al., 1973; Chappel, 1993a). Immunological means include ELISA, Western blot immunoelectrophoresis, immunodiffusion techniques, rocket gel electrophoresis, radio-immunoassay techniques, amongst others. Molecular-biological means include nucleic acid hybridisation, nucleic acid sequencing techniques, polymerase chain reaction techniques and variations thereto, amongst others. Those skilled in the relevant art will be aware of variations and optimisations which may be applied to these procedures, in typing the leptospire of the invention.

The invention described according to this aspect extends to said isolated bacterium when provided as a culture in liquid or solid form, such as but not limited to a glycerol stock, stab, slope, plate or in a freeze-dried or otherwise-dried form, for example on a membraneous filter or paper disc.

A second aspect of the present invention is directed to a method of isolation of the pathogenic *Leptospira* serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or an immunologically-cross-reactive or a genetically-cross-reactive serovar or a derivative bacterium thereof comprising the steps of:

1. Collection of human or animal tissue from a host organism which is infected with said pathogen;
2. Homogenisation of said tissue in homogenisation medium suitable for maintaining the integrity of said pathogenic bacterium; and
3. Culture of said tissue containing said *Leptospira* bacterium in a culture medium for a time sufficient to allow bacteria to grow to the required density.

The culture medium may be any medium appropriate for the purpose of culturing a *leptospira* bacterium, which are generally known to those skilled in the art, for example EMJH medium described by Chappel (1993b).

According to this aspect of the present invention, a person skilled in the art would be aware that said culture of *Leptospira* may require sub-culturing at certain intervals, in order to maintain the viability of the culture. Such sub-culturing serves to replace nutrients in the media which are essential to viability and/or growth of the bacterium. If sufficient cycles of sub-culturing are carried out, this will eventually produce a bacterial culture which is essentially free of contaminating tissue derived from the host organism.

Preferably, the human or animal tissue from which said pathogen is obtained is blood or tissue of the urogenital tract selected from the list comprising bladder, kidney, uterus or fallopian tube, testes or ovaries or, alternatively, from liver or lung tissue, or from body fluids or exudates such as urine or cerebrospinal fluid, amongst other sources. More preferably, said tissue originates from a preferred host of the pathogenic leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular a human or a livestock or companion animal selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

It will be understood by those skilled in the art that there are a range of suitable homogenisation media which may be used, the only requirement being that the particular homogenisation medium used maintains the bacterium in a viable state such that sufficient viable cells exist in the homogenate to establish a viable culture.

The present invention extends to the use of any suitable homogenisation medium in the isolation of the subject leptospire including, for example, media containing phosphate-buffered albumin.

Preferably, the culture medium contains in addition to 8-azaguanine, 5-fluorouracil and at

least one antibiotic selected from the list comprising a rifamycin, macrolide polyene or quinoline antibiotic, amongst others.

Rifamycin antibiotics are high substituted macrocyclic compounds which are active against Gram-positive bacteria and certain Gram-negative bacteria but to which spirochaete bacteria including *Leptospira* bacteria are resistant. Rifamycins specifically inhibit eubacterial DNA-dependent RNA polymerase, binding to the β-subunit and inhibiting transcription.

The macrolide polyenes are characterised by a substituted or unsubstituted lactone ring containing a rigid, lipophilic region of unsubstituted *trans*-conjugated double bonds and a flexible, hydrophilic hydroxylated region. Macrolide polyenes interact with sterols in the cytoplasmic membrane, causing leakage of ions and small molecules. Macrolide antibiotics are not effective against bacteria which do not contain sterols in their membranes. Macrolide antibiotics are microbistatic at low concentrations or microbicidal at higher concentrations against yeast and other fungi and against protozoa which contain sterols in their membranes. Preferred macrolide polyenes are selected from the list comprising amphotericin, aureofungin, candicidin B, etruscomycin, filipin, hamycin, hystatin, perimycin, pimaricin and trichomycin amongst others.

Quinoline antibiotics contain a substituted 4-quinoline ring and are primarily active against Gram-negative bacteria. Preferred quinoline antibiotics include but are not limited to antibiotics selected from the list comprising naladixic acid, cinoxacin, oxolinic acid, pipemidic acid, ciprofloxacin, enoxacin, norfloxacin, ofloxacin or perfloxacin, amongst others.

The list of antibiotics provided for the isolation of a *Leptospira* bacterium according to the present invention is not exhaustive and the person skilled in the art will appreciate that alternative or additional antibiotics may be used. The person skilled in the art will also be aware that the tissue from which the pathogenic leptospire is to be isolated may contain

several contaminating microorganisms in addition to said *Leptospira* bacterium and the particular combination of antibiotics selected for use will vary depending upon the nature of the contaminating microorganisms present. The present invention clearly contemplates the use of additional antibiotics in the culture media used for the isolation of said pathogenic *Leptospira* bacterium.

In a particularly preferred embodiment, the present invention provides a method of isolation of a pathogenic *Leptospira* bacterium as hereinbefore described wherein said bacterium is a serovar which has been deposited with AGAL on 15 November, 1995 and assigned AGAL Accession Number N95/69684.

In a particularly preferred embodiment, said method is useful for the isolation of the pathogenic *Leptospira* bacterium deposited with AGAL on 15 November, 1995 and assigned AGAL Accession Number N95/69684.

A third aspect of the present invention provides agents and chemical compositions for use in the isolation of the pathogenic leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof, essentially according to the methods described herein.

In a preferred embodiment, the agent or chemical composition is a culture medium for the selective growth of the leptospire of the invention.

According to this aspect of the present invention, the agent or chemical composition may be in powdered, liquid, tablet, pellet, capsule or other form.

The present invention extends to an agent or chemical composition as described herein, wherein said agent or chemical composition is used for, or intended to be used for the isolation, detection, purification, culture or storage of a pathogenic microorganism, preferably a pathogenic bacterium, more preferably a pathogenic *Leptospira* bacterium in

particular the leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* such as the strain deposited under AGAL Accession Number N95/69684 or a derivative bacterium thereof.

A fourth aspect of the present invention provide an isolated nucleic acid molecule comprising a sequence of nucleotides which corresponds to, or is complementary to a sequence of nucleotides which corresponds to the 16S rRNA gene or a derivative, homologue or analogue thereof of the pathogenic *Leptospira* bacterium of the present invention.

Reference herein to "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'-untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred rRNA genes may be derived from a naturally-occurring serovar, in particular the rRNA gene of serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, by standard recombinant techniques. Generally, a rRNA gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions. Nucleotide insertional derivatives of a rRNA gene of the present invention include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotide are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place.

Accordingly, the isolated nucleic acid molecule of the present invention may comprise genomic DNA, cDNA, RNA or a synthetic oligonucleotide molecule in single-stranded or double-stranded form. The present invention further extends to conformational isomers of such molecules.

Preferably, the isolated nucleic acid molecule comprises a nucleotide sequence which is at least 80% identical to the nucleotide sequence set forth in any one of SEQ ID NOs:1-2 or 4-7 or a complement or a derivative, homologue or analogue thereof.

In an alternative embodiment, the isolated nucleic acid molecule at least comprises a nucleotide sequence which is at least 80% identical to the nucleotide sequence 5'-TGTTGGA-3' (SEQ ID NO:4) or at least 90% identical to the nucleotide sequence 5'-TTTGATA-3' (SEQ ID NO:5) or at least 90% identical to the nucleotide sequence 5'-TGTTGGA(N)$_8$TTTGATA-3' (SEQ ID NO:6) or a complement, or a derivative, homologue or analogue thereof, wherein N is any nucleotide residue.

More preferably, the isolated nucleic acid molecule comprises a nucleotide sequence which is at least about 85% identical to the nucleotide sequence:

5'-TGTTGGATCACAAGATTTGATA-3' (SEQ ID NO:7)

or a complement, or a derivative, homologue or analogue thereof.

Alternatively or in addition, the isolated nucleic acid molecule is capable of hybridising under high stringency conditions to any one of the nucleotide sequences described *supra* or to its complementary nucleotide sequence, or a derivative, homologue or analogue thereof.

For the present purpose, homologues of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as or at least 80% identical to a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide

substitutions, insertions, deletions, or rearrangements.

Analogues of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

Derivatives of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

Preferred homologues, analogues and derivatives comprise at least about 5-15 nucleotides in length and more preferably at least about 15-30 nucleotides in length and are at least about 80% identical to the nucleotide sequences of the invention described herein. Alternatively, the homologues, analogue and derivatives described herein may further comprise a nucleotide sequence which is at least about 90% identical, more preferably at

least about 95% identical, even more preferably at least about 97% identical and still more preferably at least about 99% identical to any one of to the nucleotide sequences of the invention described herein. Particularly preferred homologues, analogues and derivatives comprise at least about 15-18 nucleotides in length derived from any one of the nucleotide sequences of the invention described herein.

For the purposes of the present invention, it is preferred that the nucleic acid molecule of the invention is the 16S rRNA genetic sequence of the leptospire which has been deposited with AGAL under Accession Number N95/69684. It will be known to those skilled in the relevant art that derivative bacteria of the deposited leptospire or bacteria belonging to the same species will generally contain 16S rRNA genetic sequences which are more closely related to the 16S rRNA of serovar hurstbridge or serogroup Hurstbridge or *L. fainei* than are the 16S rRNA genetic sequences obtained from more distantly-related, or unrelated *Leptospira*. As a consequence, the genetic sequence of the present invention is at least useful in determining whether or not a pathogenic *Leptospira* bacterium is closely related to serovar hurstbridge or serogroup Hurstbridge or *L. fainei*. Said genetic sequence is also useful in the isolation of genetic sequences from serovars of *Leptospira* which are closely-related to serovar hurstbridge or serogroup Hurstbridge or *L. fainei*.

The person skilled in the art will be aware of nucleic acid hybridisation techniques which may be used to identify leptospires which are related to serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular the various hybridisation stringencies which may be employed in such an identification procedure. For the purposes of the defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C. Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification, of the parameters affecting hybridisation between nucleic acid molecules, reference is found in pages 2.10.8 to 2.10.16 of Ausubel *et al* (1987), which is

herein incorporated by reference.

The person skilled in the art will appreciate that the nucleic acid molecules of the present invention may correspond to the naturally-occurring sequence or may differ by one or more nucleotide substitutions, deletions and/or additions. Accordingly, the present invention extends to rRNA genes and any isolated, synthetic or recombinant genes, oligonucleotides, mutants, derivatives, parts, fragments, homologues or analogues thereof which are at least useful as, for example, genetic probes, or primer sequences in the enzymatic or chemical synthesis of said gene, or in the generation of immunologically interactive recombinant molecules, or in the isolation or detection of a pathogenic *Leptospira* bacterium.

In a particular preferred embodiment, the serovar hurstbridge or serogroup Hurstbridge or *L. fainei* rRNA genetic sequence or a derivative, homologue or analogue thereof, is employed to identify similar genes from cells, tissues, or organ types of a host organism, in particular, the cells, tissues or organs of the urogenital tract including the bladder, uterus, fallopian tubes or kidney, or body fluids or exudates such as urine or cerebrospinal fluid, amongst others, which may be infected with a pathogenic *Leptospira* bacterium.

According to this embodiment, there is contemplated a method for identifying a related rRNA genetic sequence in a host organism which may be infected with a pathogenic *Leptospira* bacterium, said method comprising contacting cellular extract or nucleic acid sample obtained from said host organism with a hybridisation effective amount of a rRNA genetic sequence or a functional part thereof derived from serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, and then detecting said hybridisation. Accordingly, this embodiment of the present invention also relates to a method of identifying a serovar of *Leptospira* which is related to leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular the leptospiral strain deposited with AGAL on 15 November, 1995 under Accession No. N95/69684.

Said rRNA genetic sequence may be labelled with a reporter molecule which is capable of

giving an identifiable signal (eg. a radioisotope such as ^{32}P or ^{35}S or a biotinylated molecule).

An alternative method contemplated in the present invention involves hybridising two nucleic acid "primer molecules" of at least 15 nucleotides in length derived from the rRNA sequence of the invention or its complementary sequence to a nucleic acid "template molecule" derived from a cell, tissue or organ of a host human or other animal being tested for the presence of a pathogenic *Leptospira* bacterium, said template molecule herein defined as a related leptospiral 16S rRNA genetic sequence, or a functional part thereof, or its complementary sequence. Specific nucleic acid molecule copies of the template molecule are amplified enzymatically in a polymerase chain reaction. Methods for the isolation of said template molecule and for the polymerase chain reaction are known to those skilled in the art.

The nucleic acid primer molecules are generally single-stranded synthetic oligonucleotides although the present invention also contemplates other primers. According to this embodiment, the nucleic acid primer molecule consists of a combination of any of the nucleotides adenine, cytidine, guanine, thymidine, or inosine, or functional analogues or derivatives thereof, capable of being incorporated into a polynucleotide molecule.

Preferably, each nucleic acid primer molecule is any nucleotide sequence of at least 15 nucleotides in length derived from, or complementary to the nucleotide sequence of serovar hurstbridge or serogroup Hurstbridge or *L. fainei* 16S rRNA or a derivative, homologue or analogue thereof. In a particularly preferred embodiment, at least one primer molecule is substantially the same as, or complementary to, nucleotide sequences set forth in SEQ ID NOs:2 and 3.

The nucleic acid template molecule may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the related genetic sequence originates from a mammalian cell, tissue or organ, optionally infected with a

pathogenic leptospiral bacterium such as serovar hurstbridge or serogroup Hurstbridge or *L. fainei*. More preferably, said mammalian cell, tissue or organ further originates from a human or a livestock or companion animal which is capable of being infected with said bacterium, in particular a livestock or companion animal selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

A further aspect of the present invention provides an immunologically interactive molecule prepared against one or more immunogens of a pathogenic *Leptospira* bacterium or derivative bacterium thereof which grows at temperatures from about 13°C to about 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a particularly preferred embodiment, the bacterium is the leptospire of the invention or a derivative bacterium thereof. In a most particularly preferred embodiment, said bacterium is the strain deposited with AGAL on 15 November, 1995 under AGAL Accession No. N95/69684 or a derivative bacterium thereof.

The term "immunologically interactive molecule" as used herein shall be taken to include polyclonal or monoclonal antibodies, or functional derivatives thereof, for example Fabs, SCABS (single-chain antibodies) or antibodies conjugated to an enzyme, radioactive or fluorescent tag, the only requirement being that said immunologically interactive molecule is capable of binding to an immunogen derived from or present in or present on the surface of a *Leptospira*, in particular serovar hurstbridge or serogroup Hurstbridge or *L. fainei*.

In an alternative embodiment, the present invention provides an immunologically interactive molecule prepared against one or more immunogens of a pathogenic *Leptospira* bacterium or derivative bacterium thereof which is capable of growing at temperatures of from about 13°C to about 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine

and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine, wherein at least one of said immunogens is a surface lipopolysaccharide molecule.

In a particularly preferred embodiment of the invention, said *Leptospira* bacterium is the serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof. Most preferably, said bacterium is the strain deposited with AGAL on 15 November, 1995 under AGAL Accession No. N95/69684 or a derivative bacterium thereof.

In a related embodiment, the immunologically interactive molecule of the present invention may be prepared against an immunogen which mimics, or cross-reacts with a B cell or T cell epitope of a surface lipopolysaccharide molecule of a pathogenic *Leptospira* bacterium, preferably serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof, more preferably the strain deposited with AGAL on 15 November, 1995 under AGAL Accession Number N95/69684.

Conventional methods can be used to prepare the immunologically interactive molecules. For example, by using the bacterial strain or serovar of the present invention or an immunogen derived therefrom, polyclonal antisera or monoclonal antibodies can be made using standard methods. As demonstrated herein, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunised with an immunogenic form of serovar hurstbridge or serogroup Hurstbridge or *L. fainei* which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a surface protein or other molecule produced by the leptospire of the invention include conjugation to carriers or other techniques well known in the art. For example, the bacterium can be administered in the presence of adjuvant. The progress of immunisation can be monitored by detection of antibody titres in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. Following immunisation, antisera can be obtained and, if desired IgG molecules corresponding to the polyclonal antibodies may be isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunised animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalising these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (1975) as well as other techniques such as the human B-cell hybridoma technique (Kozbor *et al.*, 1983), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, 1985), and screening of combinatorial antibody libraries (Huse *et al.*, 1989). Hybridoma cells can be screened immunochemically for production of antibodies which are specifically reactive with the polypeptide and monoclonal antibodies isolated.

As with all immunogenic compositions for eliciting antibodies, the immunogenically effective amounts of the immunogen must be determined empirically. Factors to be considered include the immunogenicity of the immunogen, whether or not it is to be complexed with or covalently attached to an adjuvant or carrier protein or other carrier and route of administration for the composition, i.e. intravenous, intramuscular, subcutaneous, *etc.*, and the number of immunising doses to be administered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The term "antibody" as used herein, is intended to include fragments thereof which are also specifically reactive with a molecule which comprises, mimics, or cross-reacts with a B cell or T cell epitope of a surface lipopolysaccharide or surface polypeptide or other molecule produced by serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular the strain deposited with AGAL under Accession Number N95/69684. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any immunogen of leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular the strain deposited under AGAL Accession Number N95/69684. Preferably, said immunogen is a surface lipopolysaccharide molecule or a molecule which mimics a continuous or discontinuous B-cell or T-cell epitope of same.

The polyclonal, monoclonal or chimeric monoclonal antibodies can be used to detect the pathogenic bacterium of the invention or a derivative bacterium thereof in various biological materials, for example they can be used in an ELISA, radioimmunoassay or histochemical tests. Said antibodies are also useful in the detection of the isolated immunogen against which they are prepared, in either impure or pure form. Thus, the antibodies can be used to test for binding to the leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, or a derivative bacterium thereof in a sample or to test for binding to the isolated immunogen or to test for binding to any molecule which cross-reacts with a B cell or T cell epitope of same.

A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilised on a solid substrate and the sample

to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time and under conditions sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule.

In this case, the first antibody is raised to an immunogen of a pathogenic *Leptospira* bacterium, wherein said bacterium is preferred to be serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a strain deposited with AGAL under Accession Number N95/69684 as described herein. More preferably, said first antibody is raised to an immunogen of said pathogenic *Leptospira* bacterium wherein, said immunogen is a surface lipopolysaccharide of the serovar hurstbridge or serogroup Hurstbridge or *L. fainei*.

The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof or alternatively, an immunogen derived from said bacteria.

In the typical forward sandwich assay, a first antibody raised against serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or an immunogen thereof is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay.

The binding processes are well-known in the art and generally consist of cross-linking, covalent binding or physically adsorption. the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25°C) to allow binding of any antigen present in the sample to the antibody. Following the incubation period. the reaction locus is washed and dried and incubated with a second antibody specific for a portion of the first antibody. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilising the target molecules in the biological sample and then exposing the immobilised target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal. a bound target may be detected by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which. by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised. however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase. amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the

corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. The term "reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in enzyme immunoassays (EIA), the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescene and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the above assays and all such variations are encompassed by the present invention.

Accordingly, a further aspect of the present invention provides a method for the detection, identification or quantification of a pathogenic *Leptospira* bacterium or derivative bacterium thereof, wherein said method comprises the steps of incubating the material or

bacteria derived therefrom with an antibody which recognises said bacteria or an immunogen derived therefrom for a time and under conditions sufficient for an antibody: immunogen or antibody; bacterium complex to form and subjecting said complex to a detecting means.

According to this aspect of the invention, the complex may be detected by using the bacterium or immunogen derived therefrom or the antibody molecule with a reporter molecule attached thereto. Alternatively, the complex may be detected by the addition of a second antibody labelled with a reporter molecule.

Preferably, the invention according to this aspect provides a method for the detection, identification or quantification of a pathogenic *Leptospira* bacterium or derivative bacterium thereof which is capable of growing in a medium as hereinbefore described, at temperatures of 13°C to 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, more preferably at least 150µg/ml 8-azaguanine, even more preferably at least 200µg/ml 8-azaguanine and most preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a most particularly preferred embodiment, this aspect of the invention and the embodiments described therein relate to a method for the detection, identification or quantification of the leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, such as the strain deposited under AGAL Accession No. N95/69684.

According to this aspect of the invention, the material or bacteria derived therefrom is in a biological tissue or organ derived from a mammalian animal which is a host for a bacterium of the genus *Leptospira*, in particular serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or derivative bacterium thereof. Preferably, said mammalian animal is a human or a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

The biological sample to be tested may be any cell, tissue or organ which is capable of being infected with a bacterium of the genus *Leptospira*, in particular a cell, tissue or organ of the urogenital tract such as kidney, bladder, fallopian tube, uterus or endometrium, testes, or a body fluid or exudate such as, but not limited to urine or cerebrospinal fluid, amongst others. The present invention also contemplates the use of blood or blood-derived products as a biological sample suitable for the detection, identification or quantification of serovar hurstbridge or serogroup Hurstbridge or *L. fainei*.

A further aspect of the present invention contemplates a kit for the rapid and convenient assay of pathogenic *Leptospira* bacterium or derivative bacterium thereof in a biological sample, wherein said bacterium is capable of growing at temperatures from about 13°C to about 37°C and/or in the presence of at least 100µg/ml 8-azaguanine, preferably at least 150µg/ml 8-azaguanine, more preferably at least 200µg/ml 8-azaguanine and even more preferably at least 250µg/ml, or up to and including a concentration of 500µg/ml 8-azaguanine.

In a particularly preferred embodiment, the present invention contemplates a kit for the rapid and convenient assay of a pathogenic *Leptospira* bacterium or derivative bacterium thereof in a biological sample, wherein said bacterium is further characterised as leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof according to any or all of the descriptions provided herein, for example the strain deposited under AGAL Accession No. N95/69684.

In one embodiment, said kit is compartmentalised to receive several first containers adapted to contain at least one immunogen each derived from the leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* and several second containers adapted to contain an antibody molecule which binds to said pathogenic *Leptospira* bacterium, derivative bacterium thereof or immunogen derived therefrom, or alternatively, said second container contains an antibody molecule which binds to serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof or immunogen derived therefrom. Preferably,

said second container contains an antibody which binds to the serovar deposited with AGAL under Accession Number N95/69684, or an immunogen derived therefrom, in particular a surface lipopolysaccharide immunogen.

According to this embodiment of the present invention, said antibody molecule is optionally labelled with a reporter molecule capable of producing a detectable signal as hereinbefore described. If the first antibody molecule is not labelled with a reporter molecule, the kit also provides several third containers which contain a second antibody which recognises the first antibody and is conjugated to a reporter molecule. The reporter molecule used in this kit may be an enzyme, a radio-isotope, a fluorescent molecule or bioluminescent molecule, amongst others.

When the kit contains a first antibody or second antibody molecule which is conjugated to a reporter molecule which is an enzyme, then said kit also provides several fourth containers which contain a specific molecule for said enzyme to facilitate detection of the immunogen: antibody complex or immunogen: antibody: antibody complex.

Optionally, the first, second, third and fourth containers of said kit may be colour-coded for ease-of-use.

In an exemplified use of the subject kit, a control reaction is carried out in which the contents of the first container are contact with the contents of the second container for a time and under conditions sufficient for an immunogen:antibody complex to form in said first container. At the same time the sample to be tested is contacted with the contents of the second container for a time and under conditions sufficient for an immunogen:antibody complex to form in said second container. If the antibody of the second container provided is not labelled with a reporter molecule, then the complexes produced in said first and second containers are contacted with the antibody of the third container for a time and under conditions sufficient for a tertiary immunogen:antibody:antibody complex to form. The immunogen:antibody complex of immunogen:antibody:antibody complex is then

subjected to a detecting means as hereinbefore described. In analysing the results obtained using said kit, the control reaction carried out in said first container should always provide a positive result upon which to compare the results obtained in said second container which contains the test sample.

In an alternative embodiment, the present invention contemplates a kit for the rapid and convenient assay of leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* or a derivative bacterium thereof in a biological sample, wherein said kit is compartmentalised to receive several first containers adapted to contain two non-complementary primer molecules of at least 10 nucleotides, preferably at least 15 nucleotides and more preferably, at least 22 nucleotides in length. According to this embodiment, it is preferred that at least one of the first primer molecules is substantially identical to a region of the nucleotide sequence set forth in any one of SEQ ID NOs:1-7, more preferably any one of SEQ ID NOs:1-2 or 4-7 or a complement, or a derivative, homologue or analogue thereof and the second of said primer molecules is substantially identical to the complement of a region of the sequence set forth in SEQ ID NO:1 or a derivative, homologue or analogue thereof. Those skilled in the art will be aware of suitable combinations of nucleic acid primer molecules for the performance of this aspect of the present invention.

In a particularly preferred embodiment, the primer molecules are utilised as primer pairs, more preferably comprising SEQ ID NOs: 2 and 3 or primers LU [SEQ ID NO:19] and rLP [SEQ ID NO:24] (Table 10) or primers C [SEQ ID NO:20] and INT rLP [SEQ ID NO:26] (Table 10) or primers which are at least 80% identical thereto.

According to this embodiment, said kit also contains several second containers adapted to contain a reaction mixture comprising buffer and salt solution either ready-for-use or in concentrated form and several third containers adapted to contain an enzyme suitable for use in a nucleic acid hybridisation reaction or a polymerase chain reaction, for example any heat stable DNA polymerase enzyme, in particular *Thermophilus aquaticus Taq*I, or similar enzyme. Optionally, the first, second and third containers of said kit maybe colour coded

for ease-of-use.

For the purposes of this embodiment of the present invention, the biological sample may be any cell, tissue, organ, body fluid or exudate of a mammalian animal which is capable of carrying a serovar of a pathogenic *Leptospira* bacterium of the invention, including for example any cell, tissue or organ of the urogenital tract, bladder, kidney, uterus, endometrium, testes or fallopian tube or a body fluid or exudate such as urine or cerebrospinal fluid, amongst others. The invention also contemplates the use of blood as a biological sample which is useful for the present purpose. Alternatively, or in addition to the foregoing examples of suitable biological samples, it is also possible to use a nucleic acid extract obtained from said cell, tissue or organ sample. Preferably, said biological sample originates from a livestock animal such as a human or a livestock or companion animal, in particular a livestock or companion animal selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

In an exemplified use of the subject kit described in this embodiment, a test sample reaction is carried out wherein the contents of the first, second and third containers are combined and a biological sample to be tested is added thereto. A negative control reaction may also be set up in which no biological sample is added to the reaction mixture. The test sample and negative control reactions are incubated for a time and under conditions sufficient for the amplification of DNA sequences which originate from the subject bacterium to occur.

A further aspect of the present invention contemplates a diagnostic test for the identification of a *Leptospira* pathogen in a biological sample using the methods, reagents and kits of the present invention as hereinbefore defined. Particularly preferred diagnostic assays are based on the serological detection of bacteria of the serogroup Hurstbridge using MAT or alternatively, the genetic detection of bacteria belonging the same species *L. fainei* using nucleic acid-based hybridisation and/or amplification reactions.

The present invention also extends to compositions comprising isolated recombinant

polypeptide immunogens derived from a leptospiral bacterium and immunologically interactive molecules thereto, such as antibodies and serum comprising same, wherein said leptospiral bacterium belongs to the same serogroup as serogroup Hurstbridge or to the same serovar as serovar hurstbridge.

Accordingly, a still further aspect of the present invention contemplates a composition comprising:

1. one or more immunogens which are immunologically cross-reactive with a cellular component of a pathogenic *Leptospira* bacterium belonging to serogroup Hurstbridge or derivative bacterium thereof; and

2. one or more pharmaceutically or veterinarially acceptable carriers, adjuvants and/or diluents.

Alternatively, the composition comprises:

1. an antibody molecule or sera comprising same which is capable of binding to one or more antigens of a pathogenic *Leptospira* bacterium belonging to serogroup Hurstbridge or derivative bacterium thereof; and

2. one or more pharmaceutically or veterinarially acceptable carriers, adjuvants and/or diluents.

The antibody molecule may be a monoclonal or polyclonal antibody, immunoglobulin fraction, Fab or recombinant single-chain antibody molecule or an immunological equivalent thereof.

Preferably, the composition according to these embodiments is a vaccine preparation. In a more preferred embodiment, said compositions induce humeral immunity against serovar hurstbridge or serogroup Hurstbridge or *L. fainei* when administered to a human or animal subject. In a most particularly preferred embodiment, said composition induces humoral immunity against the leptospire deposited with AGAL under Accession Number N95/69684.

In a preferred embodiment, the immunogen or antigen according to this aspect of the invention is immunologically cross-reactive with a bacterium characterised according to any or all of the descriptions provided herein as leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular the leptospiral strain deposited with AGAL under Accession Number N95/69684.

In a more preferred embodiment, at least one of said immunogens or antigens is a surface lipopolysaccharide.

According to this aspect of the present invention, the immunogen component of an effective composition may also comprise a complete, attenuated leptospiral serovar hurstbridge or serogroup Hurstbridge or *L. fainei* which has been pre-treated to render it non-infectious and predominantly asymptomatic. Methods for attenuating said leptospiral serovar include, but are not limited to formalin-killing, heat-killing, irradiation or genetic modification to remove genetic material related to pathogenesis.

The compositions of the present invention are contemplated to exhibit excellent therapeutic activity, for example, in the prevention of diseases associated with infection by leptospiral pathogens such as serovar hurstbridge or serogroup Hurstbridge or *L. fainei*, in particular reproductive disease. Preferably, said composition is effective in mediating an immune response when administered to a mammalian animal, in particular to a human or a livestock or companion animal, such as a livestock or companion animal selected from the list comprising pigs, bovines, sheep, goats, horses, deer, alpacas, dogs or cats, amongst others.

The term "mediating an immune response" as used herein is defined in its broadest context to include the elicitation of T-cell activation by an immunogen and/or the generation, by B-cells, of neutralising antibodies which cross-react with one or more molecules encoded by a pathogenic serovar of *Leptospira* belonging to serogroup Hurstbridge as described herein or a derivative bacterium thereof. In particular, said neutralising antibodies cross-

react with one or more molecules encoded by serovar hurstbridge or derivative bacterium thereof.

The composition may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules). Depending on the route of administration, the immunogens contained therein may be required to be coated in a material to protect them from the action of enzymes, acids and other natural conditions which otherwise might inactivate said immunogen. In order to administer the composition by other than parenteral administration, they will be coated by, or administered with, a material to prevent its inactivation. For example, the immunogen may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. "Adjuvant" as used herein is to be taken in its broadest sense and includes any immune-stimulating compound such as a cytokine. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes.

The composition of the present invention may also be administered parenterally or intra peritoneally. Dispersions of the immunogen component can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of

microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the immunogen of the present invention in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by heat-sterilisation, irradiation or other suitable sterilisation means. Generally, dispersions are prepared by incorporating the various sterilised active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When immunogens are suitably protected as described above, the protected immunogen may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral administration, the protected immunogen may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups,

wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of immunogen in such compositions is such that effective immunisation will be achieved with between one and five doses of said vaccine.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the immunogen of the present invention may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable or veterinarially acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated: each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutically or veterinarially acceptable carrier.

For the purposes of exemplification only, the present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a copy of a photographic representation of an electron micrograph of a bacterium belonging to serovar hurstbridge or serogroup Hurstbridge or *L. fainei*. *leptospira* strain BUT6 was examined by transmission electron microscopy using a Philips CM12 STEM electron microscope, employing negative staining with 2% phosphotungstic acid at a final magnification of 9,450X. The bar shows 1µm. Bacterial cells are approximately 12µm in length and 0.2µm in diameter and exhibit the typical helical or spiral morphology which is characteristic of *Leptospira*.

Figure 2 is a schematic representation showing the alignment of a 200bp region of the rRNA gene sequences of *L. fainei* (derived from SEQ ID NO:1) to the rRNA gene sequences of other *Leptospira* serovars, including *L. inadai* serovar lyme (SEQ ID NO:8) , *L. meyeri* serovar ranarum (SEQ ID NO:9), *L. weilii* serovar celledoni (SEQ ID NO:10), *L.santarosi* serovar shermani (SEQ ID NO:11), *L. borgpetersenii* serovar javanica (SEQ ID NO:12), *L. kirschneri* serovar cynopteri (SEQ ID NO:13) and *L. interrogans* serovar icterohaemorrhagiae (SEQ ID NO:14). Asterisks indicate variable nucleotide residues. Base numbering is indicated at the top of the alignment.

EXAMPLE 1

General strategy and selection of swine herds for culture

Three swine herds in Victoria and New South Wales were selected for culture, all serologically positive to *Leptospira interrogans* serovar bratislava. *Leptospira* bacteria had been visualised in each herd by immunofluorescent staining of tissues, despite a lack of serological evidence of infection with the known Australian pig leptospiral serovars, pomona and tarassovi. Cultures were established from uterus, fallopian tube and kidney of each animal. The objective was to maximise the chance of isolating serovar bratislava by culturing from both baconer-age gilts and sows.

The sample size was limited to 30 cultures for each animal or less. The culture program involved the use of an initial 24 culture tubes for each animal: three tissues, four antibiotic combinations, and two dilutions.

EXAMPLE 2

Collection of tissues for culture

Swine herds were selected according to Example 1. Tissues for culture were collected from animals at the Hurstbridge abattoir (Herds A and B) and the Altona abattoir (Herd C). Bladders were tied off with cable ties at the point of removal and uterus with fallopian tubes, and kidneys, were collected in sterile bags. Blood was also collected at the point of slaughter and matched with tissue samples. Pigs were identified at the point of slaughter by individual tattoo.

EXAMPLE 3

Bacterial Cultures

Tissue samples obtained as described in the preceding Examples were processed as soon as possible to limit the degree of autolysis. Fallopian tube segments, uterus endometrial scrapings and kidney samples were homogenised in phosphate-buffered albumin to protect leptospires then diluted to a final concentration of 1:100 prior to inoculating two 7.5 ml volumes of culture medium with one and five drops respectively. Samples were incubated at 30°C for up to 6 months and examined at intervals of approximately two to three weeks. Four different formulations of Tween 40/80 semisolid media were used, with different combinations of antibiotics, according to a matrix shown in Table 1.

TABLE 1

Matrix of antibiotic combinations used in the culture program designed to isolate *Leptospira interrogans* serovar bratislava.

Medium	5-Fluorouracil	Rifampicin	Amphotericin B	Naladixic Acid
M1	100µg/ml	NONE	NONE	NONE
M2	200µg/ml	NONE	NONE	NONE
M3	300µg/ml	NONE	NONE	20µg/ml
M4	100µg/ml	10µg/ml	2µg/ml	NONE

Cultures were established from 27 sows and gilts. 24 of which were from the three target herds (Table 2). Leptospires were observed in six cultures derived from five animals in two of the three target herds (B and C). Isolation was achieved in five cases. as shown in Table 3.

Structures similar to non-motile leptospires were observed in several cultures from herd A and appeared typical of bratislava when first observed in cultures. However. no motile leptospires developed from these cultures and isolation was not achieved. The identity of these possible leptospires could not be confirmed.

Four isolates of an organism from three sows were obtained from herd B. Isolates from herd B appeared as typical leptospires under both dark ground microscopy and transmission electron microscopy. Leptospires were also observed in two cultures from Herd C but only one isolate was obtained (Table 3).

EXAMPLE 4

Microscopic agglutination test (MAT)

The microscopic agglutination test (MAT) (Cole *et al.*, 1973) was performed using serovar hurstbridge isolate No. 6 (Table 3) as the live or reference antigen. Sera were typically

tested at final dilutions (including antigen) from 1/32 to 1/256 or above. Rabbit antiserum to each serovar tested was included on each microtitre plate as a positive control. Titres were expressed as the reciprocal of the final serum dilution (including the volume of antigen) at which agglutination of 50% or more was observed.

The herd B isolate (Table 3) characterised according to its agglutination with antisera against a range of leptospiral pathogens. These isolates were not agglutinated to high titre by antisera against bratislava, pomona, tarassovi, hardjobovis, copenhageni or a number of other pathogenic serovars. The herd B organism was found to autoagglutinate strongly, and the results of these agglutination experiments were therefore difficult to read.

Isolate 1 from herd B was sent to the International Leptospirosis Reference Laboratory in Brisbane, Australia for confirmation of lack of agglutination by antisera to known leptospiral pathogens. It was also demonstrated that the isolate grew persistently at 13 °C, and in the presence of 8-azaguanine, implying that it was a saprophyte and not a pathogen.

The isolate from Herd C failed to agglutinate with antisera to a number of known pathogenic leptospires (Table 4). However it agglutinated to high titre with rabbit antiserum raised against the Herd B isolate (Table 4). This indicates that it is probably the same organism. The Herd C isolate showed no autoagglutination when first obtained, unlike the isolates from Herd B.

TABLE 2

Pigs from which tissues were cultured in an effort to isolate serovar bratislava.

Pig Number	Abattoir	Type and Age of Pig	Herd of Origin	Date of Culture
1	Hurstbridge	Gilt	A	20/10/93
2	Hurstbridge	Gilt	A	26/10/93
3	Hurstbridge	Gilt	A	26/10/93
4	Hurstbridge	Gilt	A	3/11/93
5	Hurstbridge	Gilt	A	3/11/93
6	Hurstbridge	Sow	A	4/11/93
7	Hurstbridge	Sow	N.S.W. herd	4/11/93
8	Hurstbridge	Sow	A	4/11/93
9	Hurstbridge	Sow	N.S.W. herd	23/11/93
10	Hurstbridge	Sow	Victorian herd	23/11/93
11[1]	Hurstbridge	Gilt	B	2/1/94
12	Hurstbridge	Gilt	B	21/1/94
13	Hurstbridge	Gilt	B	21/1/94
14	Hurstbridge	Gilt	B	21/1/94
15	Hurstbridge	Gilt	B	21/1/94
16[2]	Hurstbridge	Sow	B	4/2/94
17[2]	Hurstbridge	Sow	B	4/2/94
18[2]	Hurstbridge	Sow	B	4/2/94
19	Hurstbridge	Sow	B	4/2/94
20	Hurstbridge	Sow	B	4/2/94
21	Hurstbridge	Sow	B	4/2/94
22	Altona	Sow	C	13/4/94
23	Altona	Sow	C	13/4/94
24[2]	Altona	Young sow	C	4/5/94
25[2]	Altona	Young sow	C	4/5/94
26	Altona	Young sow	C	4/5/94
27	Altona	Young sow	C	4/5/94

[1]Discarded early as cultures incorrectly inoculated.

[2]Leptospiral Isolates were obtained from these pigs. See Table 3.

TABLE 3
Observation of Leptospires in Cultures.

Number	Herd	Pig No.	MAT Titre to bratislava	Pig Type	Date Cultured	Date First Observed	Weeks of Culture	Tissue	Isolated	Remarks
1	B	16	128	Sow	4/2/94	16/2/94	2	Uterus	Yes	These isolates appear to be identical.
2	B	16	128	Sow	4/2/94	20/2/94	2	Kidney	Yes	
3	B	17	32	Sow	4/2/94	23/3/94	7	Kidney	Yes	
4	B	18	64	Sow	4/2/94	26/4/94	11	Uterus	Yes	
5	C	25	<32	Young sow	4/5/94	28/6/94	8	Uterus	No	Typical leptospires, lost in culture.
6	C	24	<32	Young sow	4/5/94	31/8/94	17	Kidney	Yes	Typical leptospires. Agglutinated by antiserum to isolate 1.

TABLE 4

Microscopic agglutination test titres given by isolate 6 from Herd C with some high titre rabbit antisera.

Rabbit Antiserum against:	Agglutination Titre
bratislava strain 834	<4
bratislava strain Jez	<4
pomona	32
tarassovi	64
isolate 1 from Herd B	≥8192

EXAMPLE 5

MAT titres in human leptospirosis patients

723 sera derived from human subjects which had been submitted to Monash University, Victoria, Australia for diagnostic leptospirosis serology were also tested by the MAT for antibodies to serovar hurstbridge or serogroup Hurstbridge. Approximately 79% of the sera were obtained from males. Most sera were believed to be derived from patients exhibiting symptoms consistent with leptospirosis.

The MAT were initially conducted at Monash University using the *Leptospira borgpetersenii* serovars ballum, hardjobovis and tarassovi and the *L. interrogans* serovars australis, copenhageni and pomona as antigens. The MAT for these serovars differed from the MAT for serovar hurstbridge or serogroup Hurstbridge as described in Example 4 in the following particulars: Firstly, agglutination was observed microscopically after transferring a loop of suspension from each well of a microtitre plate onto a microscope slide. Secondly, the first serum dilution in the dilution series was 1/50.

In the present study, MAT for serovar hurstbridge or serogroup Hurstbridge was performed as described in Example 4 using these 723 serum samples.

Additionally, a control group of sera obtained from 62 staff at the Victorian Institute of Animal Science (VIAS), Victoria, Australia was also subjected to MAT for serovar hurstbridge or serogroup Hurstbridge antibodies. The 62 control sera came from 27 males and 35 females.

As shown in Table 5, MAT titres in the two groups of sera were strikingly different. Of the 723 diagnostic sera tested, 7.2% of sera had titres of >512 and 13.4% of sera had titres of >128. In contrast, all 62 sera in the control group from VIAS had MAT titres of 32 or less. The difference between the groups in titres of >128 was highly significant ($\chi^2 = 9.55$, df=1.0; $p < 0.01$). Analysis of postal area codes showed that patients with MAT titres to serovar hurstbridge or serogroup Hurstbridge came predominantly from dairying and pig-producing areas of Victoria.

The prevalence of high titres to each serovar in the diagnostic sera is shown in Table 6. About 7% of the sera gave MAT titres of ≥400 to serovar hardjobovis and a similar percentage gave MAT titres of ≥512 to serovar hurstbridge or serogroup Hurstbridge. In contrast, there were far fewer titres of ≥400 to the other serovars.

TABLE 5

MAT titres to serovar hurstbridge in human sera submitted for leptospirosis diagnostic testing compared with a control population

MAT titre	Test Group			Control Group		
	Male	Female	Total	Male	Female	Total
≤32	448	129	577	28	35	62
64	37	12	49	0	0	0
128-256	38	7	45	0	0	0
≥512	45	7	52	0	0	0
Total	568	155	723	28	35	62

TABLE 6

MAT titres of ≥400 to different leptospiral serovars in 723 human sera submitted for leptospirosis diagnostic testing.

Serovar	No sera with titres ≥400	% sera with titres ≥400
australis	1	0.1
ballum	2	0.3
copenhageni	1	0.1
hardjobovis	49	6.8
hurstbridge (>512)	52	7.2
pomona	0	0.0
tarassovi	3	0.4

EXAMPLE 6

Relationship between sow reproductive performance and titres to serovar hurstbridge or serogroup Hurstbridge

A study was performed of the relationships between reproductive performance and titres to serovar hurstbridge or serogroup Hurstbridge in a New South Wales herd. Serum samples were obtained at random from a total of 468 mixed parity sows, and serum samples were tested by the MAT for serovar hurstbridge or serogroup Hurstbridge (Example 4). Titres obtained were compared with the full reproductive histories of the sampled animals. The 468 animals sampled had been mated a total of 1484 times. The outcomes of different matings from the same sow were related to the same serological result.

Table 7 demonstrates a highly significant association between MAT titres to serovar hurstbridge and returns to service in sows in Herd B (Examples 1 and 2). Overall, sows with titres to hurstbridge were significantly more likely to return to service than serologically negative sows, an overall difference of 4.3% in farrowing rate. However, a

more detailed analysis of the data presented in Table 7 shows that the relationship involves far more returns to service when titres are 32-64, but some improvement with titres of 128 or above, possibly indicating that higher titres of serovar hurstbridge or serogroup Hurstbridge may be protective. These results are not an effect of parity, because a separate analysis conducted by the inventors has found no significant relationship between parities and hurstbridge titres.

TABLE 7

Relationship between MAT titres to serovar hurstbridge and returns to service in a New South Wales herd (Herd B) [1]

MAT hurstbridge	Farrowed	Returned	Total	% Farrowed
<32	591	80	671	88.1
32-44	330	78	408	80.9
≥128	351	54	405	86.7
Total	1272	212	1484	

1. $\chi^2 = 11.15$; df=2.0; 0.01>p> 0.00

An additional study was performed to demonstrate the relationship between reproductive performance and titres to serovar hurstbridge or serogroup Hurstbridge in a Victorian herd of animals (Herd A in Examples 1 and 2). A total of 165 mixed parity Large White/Landrace sows were randomly selected as they entered the farrowing shed. Sera from blood samples collected between one week before and one week after farrowing were analysed using the MAT for serovar hurstbridge or serogroup Hurstbridge as described in Example 4. Titres of ≥4 to serovar hurstbridge or serogroup Hurstbridge were detected for 41 sera (25 % of total sera analysed).

Foetal deaths *in utero* (10 days or more before full term) were significantly more frequent ($p<0.075$) in the Victorian herd, in animals having higher MAT titres to serovar hurstbridge or serogroup Hurstbridge (Table 8). Additionally, the mean interval from weaning to first

mating was significantly longer ($p<0.01$) in animals of this group having higher titres to serovar hurstbridge or serogroup Hurstbridge (Table 9).

TABLE 8

Relationship between MAT titres to serovar hurstbridge or serogroup Hurstbridge and percentage of foetal deaths, in sows with completed pregnancies.

MAT titre hurstbridge	<32	32	64	128
Mean % foetal deaths	3.5	2.1	5.3	25.0
Number of sows	53	35	24	4

TABLE 9

Increased weaning-to-mating interval associated with MAT titres of ≥64 to serovar hurstbridge or serogroup Hurstbridge.

MAT titre	<64	≥64
Weaning to mating (days)	5.0	5.7

EXAMPLE 7

Extracting DNA of Pathogenic Leptospires from Pig Kidney for Polymerase Chain Reaction

Five percent suspensions of Chelex 100 resin (Bio-Rad, 100-200 mesh sodium form) were prepared by adding resin to sterile distilled water while stirring, then autoclaved.

Samples of approximately 0.2 g of kidney were treated for 5 min. in a stomacher, with 2 ml of sterile phosphate buffered saline. A 0.5 ml volume of the resulting suspension was removed to a microfuge tube, and 50 μl was transferred to another tube containing 200μl of Chelex 100 suspension. The second tube was vortexed (5 sec.) and incubated at room temperature for 30 min., vortexed again and incubated at 100°C for 8 min., vortexed again and centrifuged in a microfuge at 13,000 r.p.m for 3 min.

The supernatant was removed and further purified by ethanol precipitation as follows: To a microfuge tube was added 10 μl 3M sodium acetate, 275 μl 100% ethanol, and 100 μl of Chelex 100 supernatant. The suspension was stored overnight at -20°C. The supernatant was removed, 500 μl 70% ethanol was added, and the resulting pellet was washed, with a further microfuging at 13,000 r.p.m. for 15 m. The supernatant was then removed, and dried by evaporation at room temperature for 1 h.

The pellet was resuspended in 40 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0).

EXAMPLE 8

Identifying Pathogenic Leptospires by Gene Sequences of PCR Products

PCR products of about 1.4 kb, corresponding to most of the rRNA gene sequences of a number of leptospiral serovars, were generated using oligonucleotide primers 27F and 1392R (Table 10) from conserved regions of the gene. The DNA products were purified using a Wizard™ PCR clean-up kit. The eluted products were then electrophoresed on 1%(w/v) low-melting-temperature agarose gels and the desired bands were excised using

a scalpel blade and further purified using a Wizard clean-up kit.

TABLE 10

Amplification and sequencing primers

PRIMER	NUCLEOTIDE SEQUENCE (5′→3′) [1]
27F [4]	CATGGATCCAGAGTTTGATCMTGGCTCAG (SEQ ID NO:16)
530F [4]	GTGCCAGCMGCCGCGG (SEQ ID NO:17)
926F [4]	AAACTYAAAKGAATTGACGG (SEQ ID NO:18)
LU [5]	CGGCGCGTCTTAAACATG (SEQ ID NO:19)
C [2]	CAAGTCAAGCGGAGTAGCAA (SEQ ID NO:20)
1392R [4]	ACGGGCGGTGTGTRC (SEQ ID NO:21)
1100R [4]	GGGTTGCGCTCGTTG (SEQ ID NO:22)
660R [3]	TTCACCGCTACACCTGGAA (SEQ ID NO:23)
519R [4]	GWATTACCGCGGCKGCTG (SEQ ID NO:23)
rLP [5]	ACCATCATCACATYGCTGC (SEQ ID NO:24)
B [2]	TTCCCCCCATTGAGCAAGATT (SEQ ID NO:25)
INT rLP [5]	TTATTTTTCCCTGCTTACTGAAC (SEQ ID NO:26)

1. A= adenine; C=cytosine; G=guanine; T=thymine; Y=C or T; R=A or G; K=G or T; M=A or C; W=A or T.
2. Primers B and C were disclosed by Merien *et al* (1992).
3. This primer was disclosed originally by Hookey (1992).
4. These primers are disclosed by Lane (1991).
5. These primers are disclosed by Perolat *et al* (1998).

Nucleotide sequences of the amplified DNAs were obtained on a Biosystems Model 373A DNA Sequencer, using overlapping forward primers (27F, 530F, 926F in Table 10) and reverse primers (1392R, 1100R, 660R, 519R in Table 10).

Nucleotide sequencing was attempted on the genes from serovars bratislava, hardjobovis, copenhageni, tarassovi and australis. A partial gene sequence was also obtained using the

27F primer for two isolates (1 and 2) of the leptospire cultured from Herd B.

Complete 16S ribosomal RNA gene sequences were obtained for serovars bratislava, hardjobovis, copenhageni and tarassovi. These were compared with published sequences from serovars pomona, canicola, icterohaemorrhagiae and several others, available through GENEBANK. A partial sequence derived for *L. biflexa* serovar patoc corresponded to a sequence in GENEBANK.

Nucleotide sequence homology data between the herd B leptospire and a number of leptospiral serovars is shown in Table 11. The results of this and more detailed comparison indicate that:

(a)the new isolate falls within the pathogenic grouping and not the saprophytic grouping of leptospires; (b)the new isolate nevertheless is not bratislava, pomona or tarassovi; and (c) the new isolate is most similar, with respect to rRNA gene sequence identity, to *L. inadai* serovar lyme.

TABLE 11

Homology of the sequence of the region of the 16S ribosomal RNA gene from base 51 to base 199 between the leptospire isolated from herd B and a number of other serovars.

Group	Species	Serovar	Percentage Homology
Pathogens	*L. interrogans*	bratislava	87.6
		pomona	90.2
		canicola	88.3
	L. inadai	lyme	96.6
Saprophyte	*L. biflexa*	patoc	75.2

In a further series of experiments to characterise the leptospire of the invention, leptospiral DNA was extracted from pig kidneys as described in Example 5 and rRNA gene sequences

were then amplified using the polymerase chain reaction (PCR) for detecting leptospiral DNA method described in Example 10. In these experiments, a positive control consisting of tissue extract comprising DNA and seeded with 10^5 /ml organisms of serovar pomona was included in each reaction series. The extracted DNA was amplified in a reaction mixture comprising 2.5 μl 10x Taq buffer with 15mM $MgCl_2$, 2.5 μl dNTPs (Promega), 0.5 μl each of forward and reverse primers (50 pmol/μl), 1 u Taq DNA polymerase (Promega, typically 5 unit/μl) 8.5 μl sterile distilled water. and 10 μl DNA sample. The primers used in the amplification reactions are listed in Table 10. PCR reactions were performed using a Perkin-Elmer GeneAmp PCR System 2400 using the following conditions: one cycle at 94°C for 3 min, 56°C for 1.5 mins 72°C for 2 min; twenty nine cycles at 94°C for 1 min, 56°C for 1.5 min, 72°C for 2 min; and one cycle at 94°C for 1 min, 56°C for 1.5 min, 72°C for 10 min. Amplification products were visualised after electrophoresis on 1%(w/v) agarose gels containing ethidium bromide. Each PCR product obtained was sequenced in the forward direction and in the reverse direction using a series of primers (Table 10). Consensus sequences were derived, using the results of both forward and reverse sequencing.

The nucleotide sequences of an approximately 200 base-pair region of the amplified rRNA genes of several leptospires, corresponding to nucleotide positions 139-334 of the *Escherichia coli* rRNA gene, were compared to identify variable regions between serovars. Of the 200 bases analysed, 25 nucleotides were found to vary among leptospires. The sequences in the region studied do not vary sufficiently for all pathogenic leptospiral serovars to be distinguished. However, the differences are sufficient to differentiate nine pathogenic species. A data base of sequence information was collected comprising the rRNA gene sequence set forth in SEQ ID NO:1 and rRNA sequences derived from the following eight representative serovars of eight leptospiral species: serovar javanica (*L. borpetersenii*) (SEQ ID NO:15), serovar lyme (*L. inadai*) (SEQ ID NO:8), serovar cynopteri (*L. kirschneri*) (SEQ ID NO:14), serovar ranarum (*L. meyeri*) (SEQ ID NO:9), serovar panama (*L.noguchii*) (SEQ ID NO:13), serovar shermani (*L. saratosai*) (SEQ ID NO:11), serovar celledoni (*L. weilii*) (SEQ ID NO:10) and serovar icterohaemorragiae (*L. interrogans*) (SEQ ID NO:15).

Each of these representative sequences were different. Homology between them varied

from 89.5% (21/200 bases different) to 99.5% (1/200 different). Serovar hurstbridge and *L. inadai* formed a group which is distinct from the other species examined, on the basis of rRNA gene sequence homology. Additionally, nucleotide sequences from these two species could be clearly differentiated from one another.

EXAMPLE 9

Polymerase chain reaction specific for pathogenic leptospires

PCR to detect pathogenic leptospires in culture samples was based on the method of detection of the 16S ribosomal RNA gene as described by Hookey (1992) using both the oligonucleotide primers described therein. It was found necessary to adjust the annealing temperatures used to achieve the published levels of specificity.

Samples for PCR were heated before testing, at 100°C for 10 minutes. The typical PCR reaction volume of 50 µl consisted of 1 µl sample, 5 µl buffer concentrate (giving final concentrations of 0.1M Tris-HCl, pH 9.0, O.5M KCl, 0. 1% gelatin, 15 mM $MgCl_2$, 1% Triton X-100), 5 µl dNTPs (each at final concentrations of 0.25 mM), 1 µl forward primer and 1 µl reverse primer at appropriate dilutions in water, 1 µl Taq DNA polymerase 1/5 in diluting buffer, and 36 µl water. The enzyme diluting buffer consisted of 10 mM Tris-HCl pH 7.5, 300 mM KCl, 1 mM DAT., 0.1 mM EDTA, 500 µg/ml bovine albumin, 50% (v/v) glycerol and 0. 1% (v/v) Triton X-100.

PCR was performed in a Perkin Elmer Cetus GeneAmp PCR System 9600. PCR conditions using the primers of Hookey (1992) were 35 cycles of 94°C (10 seconds), 59°C (10 seconds) and 72°C (10 seconds).

PCR was shown to be a reproducible method for detecting leptospires in culture. After modification of annealing conditions, PCR using the primers of Hookey (1992) detected all five isolated strains of serovar hurstbridge (Table 3) and the pathogenic leptospires bratislava, tarassovi and pomona. However, *Leptospira biflexa* serovar patoc, a representative saprophytic leptospire, was negative in this assay.

EXAMPLE 10

Polymerase chain reaction for detecting leptospires in serum

Oligonucleotide primers were designed to allow the amplification of part of the 16S ribosomal RNA (rRNA) gene from leptospira samples or isolated nucleic acid samples derived therefrom. A nested PCR was used to maximise sensitivity. Nested PCR is well-known to those skilled in the art and the general strategy is described for Example by McPherson *et al* (1991). The particular nested PCR strategy of the invention involved the use of two amplification reactions in sequence, wherein the first amplification reaction used primers specific for pathogenic leptospires, for example the primers described by Hookey (1992) and more particularly the primers LU (SEQ ID NO: 19) and rLP (SEQ ID NO: 24) (Table 10) to amplify rRNA sequences from crude nucleic acid or tissue samples comprising same and the second amplification reaction further amplified the rDNA obtained from the first reaction using internal primers specific for the genus *Leptospira*. A positive control, consisting of tissue extract or crude nucleic acid sample seeded with 10^5/ ml serovar pomona organisms, was included in each amplification series.

The PCR reaction mixture consisted of 2.5 µl 10X Taq buffer containing 15mM $MgCl_2$, 2.5 µl dNTP mixture comprising dATP,dCTP, dGTP and TTP (Promega), 0.5 µl of each primer at a concentration of 50 pmol/µl, 1 unit Taq DNA polymerase (Promega, diluted to 2 unit/µl), 8.5µl sterile distilled water and 10 µl sample. PCR reactions were performed in a Perkin-Elmer Gene Amp PCR System 2400. Amplification conditions were as follows: one cycle at 94°C for 3 min, 56°C for 1.5 mm, 72°C for 2 min; twenty nine cycles at 94°C for 1 min, 56°C for 1 .5 min, 72°C for 2 min; and one cycle at 94°C for 1 min, 56°C for 1.5 min, 72°C for 10 min.

The product of the first PCR reaction was diluted 1/10 in sterile distilled water, and 2.5 µl of diluted sample was included in a similar amplification reaction in a total volume of 25µl as before, using primers C (SEQ ID NO: 20) and INT rLP (SEQ ID NO: 26) (Table 10). Amplification conditions for the second reaction were similar to the initial round, however annealing reactions were at 61°C instead of 56°C.

The PCR products were subjected to electrophoresis on 1% (w/v) agarose gels containing ethidium bromide to detect a product of the predicted size. Amplified DNA was visualised by ultraviolet illumination after electrophoresis .

EXAMPLE 11

PCR specific for serovar hurstbridge or serogroup Hurstbridge or *L. fainei*

Cultures of five isolated strains of the serovar hurstbridge/ serogroup Hurstbridge/ *L. fainei* isolates described in Table 3 and of seven pathogenic leptospiral species were grown in EMJH medium and adjusted to a concentration of 2 x 10^8 organisms/ml. DNA was extracted by the silica absorption method of Boom *et al* (1990) and during this process a volume of 100 µl of culture was reduced to 25 µl, of which 5 µl was tested in the PCR reaction. Thus, approximately 4 x 10^6 organisms were tested in each PCR reaction.

A PCR was performed with oligonucleotide primers selected to detect specifically serovar hurstbridge or serogroup Hurstbridge rDNA sequences. The forward oligonucleotide primer (SEQ ID NO:2) corresponded to a region of the hurstbridge 16S ribosomal RNA gene which differed from that of other leptospires with which it was compared. The reverse oligonucleotide primer (SEQ ID NO:3) was as designed by Hookey (1992) and is one of a pair of primers used for a PCR test specific for pathogenic leptospires (Example 9).

The typical PCR reaction volume of 50 µl consisted of 5 µl sample, 5 µl of buffer concentrate giving final concentrations of 0.1M Tris-HCl, pH 9.0, 0.5M KCl, 1% Triton X-100, 20mM $MgCl_2$), 5 µl of dNTPs (each at a final concentration of 0.2 mM), 1 µl forward primer and 1 µl reverse primer at appropriate dilutions in water (each 50 pM), 5 units Taq DNA polymerase, and water to make up the volume.

PCR was performed in a Perkin Elmer Cetus GeneAmp PCR System 9600. The PCR conditions were as follows: a) one cycle of 94°C for 3 minutes, 63°C for 1.5 minutes. 72°C for 2 minutes; b) 29 cycles of 94°C for 1 minute, 63°C for 1.5 minutes, 72°C for 2 minutes; c) a further 10 minutes held at 72°C at the end of the reaction.

The PCR products were subjected to electrophoresis on 1% (w/v) agarose gels containing ethidium bromide to detect a product of the predicted size. Amplified DNA was visualised by ultraviolet illumination after electrophoresis . Results obtained are shown in Table 12.

TABLE 12

Results of PCR Specific for Serovar hurstbridge

Organism Tested	PCR Result
Serovar hurstbridge (5 strains)	Positive
Leptospira interrogans serovar pomona	Negative
Leptospira borgpetersenii serovar tarassovi	Negative
Leptospira noguchi serovar panama	Negative
Leptospira kirschneri serovar grippotyphosa	Negative
Leptospira inadai serovar lyme	Negative
Leptospira weillii serovar cellodoni	Negative
Leptospira santarosai serovar varela	Negative

This PCR reaction did not detect any serovar or serogroup other than serovar hurstbridge or serogroup Hurstbridge or *L. fainei*.

EXAMPLE 12

Passive vaccination of pigs

Fifteen piglets of approximately 4 weeks of age were acquired on 13 November 1997 and separated into three groups of five, each in an elevated pig pen in the same room. The three groups comprising 5 pigs each were administered with the following preparations:

(Group A): 5 ml immune serum which was MAT positive for serovar hurstbridge (MAT titre 256) and derived from a pig that had been administered repeatedly with serovar hurstbridge;

(Group B): 5 ml non-immune pig serum which is MAT negative for serovar hurstbridge (serum control); and

(Group C): no serum (untreated control).

Passive vaccination was performed on 24 November 1997 and piglets were subsequently challenged intraperitoneally with $\geq 10^8$ serovar hurstbridge organisms on 25 November (Day 0). Blood and urine were collected at intervals between Day 1 and Day 10.

Evidence of infection by serovar hurstbridge was determined by testing serum for the presence of leptospiral DNA, as described in Example 10. Additionally, urine was examined under a dark ground microscope for the presence of leptospires. Attempts were made to culture leptospires from urine samples, by inoculating 3 drops of urine into 5 ml of EMJH medium and performing serial dilutions of 3 drops into 5 ml of medium therefrom and finally, examining the cultures under a dark field microscope after approximately 1-2 weeks of culturing.

Results are presented in Table 13. None of the five pigs vaccinated with immune serum (i.e. Group A) showed evidence of infection as determined using PCR and light microscopy of cultured samples, however three out of five pigs in the control showed evidence of infection following challenge with serovar hurstbridge.

To further characterise the passively immunised animals, serological data were obtained using MAT (Example 4) to determine whether leptospiral infection had occurred in piglets. At 10 and 20 days post challenge, all ten control piglets (Groups B and C *supra*) had at least one titre to serovar hurstbridge in the range 32 to 512, indicating that these animals were infected (Table 14). In marked contrast, only one of the five passively-vaccinated piglets (Group A) developed a MAT titre of 32 or above (Table 14). Therefore, passive vaccination suppressed the serological evidence of infection.

TABLE 13

Evidence of infection of passively-vaccinated piglets and control piglets.

GROUP	TREATMENT	PIG	SERUM PCR DAY 1	SERUM PCR DAY 10	URINE MICROSCOPY DAY 1	URINE CULTURE DAY 3	EVIDENCE OF INFECTION
A	Immune serum	R1	-	-	-	-	No
	Immune serum	R2	-	-	-	-	No
	Immune serum	R3	-	-	-	-	No
	Immune serum	R4	-	-	-	-	No
	Immune serum	R5	-	-	-	-	No
B	Negative serum	Y1	-	-	-	-	No
	Negative serum	Y2	-	-	+	-	Yes
	Negative serum	Y3	-	-	-	-	No
	Negative serum	Y4	-	-	+	-	Yes
	Negative serum	Y5	-	-	+	+	Yes

GROUP	TREATMENT	PIG	SERUM PCR DAY 1	SERUM PCR DAY 10	URINE MICROSCOPY DAY 1	URINE CULTURE DAY 3	EVIDENCE OF INFECTION
C	No serum	P1	+	-	+	-	Yes
	No serum	P2	-	-	-	-	No
	No serum	P3	+	-	-	-	Yes
	No serum	P4	-	-	-	-	No
	No serum	P5	-	+	+	-	Yes

TABLE 13CONT.

TABLE 14

Serological evidence of infection of passively vaccinated piglets and control piglets

	MAT Titres of Serum for serovar hurstbridge					
Animal	**Day 0**	**Day 1**	**Day 3**	**Day 6**	**Day 10**	**Day 20**
Group A:						
R1	0	0	0	0	trace	trace
R2	0	0	0	trace	512	512
R3	0	0	0	0	0	0
R4	0	0	0	0	0	0
R5	0	0	0	0	0	0
Group B:						
Y1	0	0	0	32	256	64
Y2	0	0	0	0	128	64
Y3	0	0	0	32	128	512
Y4	0	0	0	0	32	0
Y5	0	0	0	0	128	128
Group C:						
P1	No sample	0	0	0	64	32
P2	0	0	0	trace	64	64
P3	0	0	0	0	64	0
P4	0	0	0	64	512	512
P5	0	0	0	0	256	256

EXAMPLE 13

Protective immunisation of pigs using a heat-inactivated vaccine against serovar hurstbridge

Twelve piglets of approximately 4 weeks of age were acquired on 13 November, 1997 and separated into two groups of six, each in a separate pen in the same room. Group A was vaccinated three times intramuscularly with an experimental vaccine containing at least 10^8 formalin-killed serovar hurstbridge organisms per dose, adjuvanted with aluminium hydroxide. Group B received a similarly prepared placebo vaccine, containing no

leptospiral organisms.

One pig in Group A died before challenge.

Pigs were subsequently challenged intraperitoneally with $\geq 10^8$ serovar hurstbridge organisms on 5 January, 1998 (Day 0). Blood was collected at intervals between Day 0 and Day 10. Sera were tested by the MAT for serovar hurstbridge or serogroup Hurstbridge, as described in Example 4.

Table 15 shows the serological results of actively vaccinated and placebo treated piglets, from the day of challenge to the day of slaughter, 10 days after challenge. From these data, it can be seen that significant antibodies against serovar hurstbridge or serogroup Hurstbridge are present in Group A at challenge, whereas the control animals have no detectable antibodies against serovar hurstbridge or serogroup Hurstbridge at challenge. Additionally, the serological response to challenge is more modest in the vaccinated group (i.e. Group A) than in the control group (i.e. Group B) which mostly possessed very high MAT titres by Day 10.

Table 16 shows the results of this experiment in terms of the increase in MAT titre in response to challenge. As shown in Table 16, the group A animals experienced a maximum 8-fold increase in MAT titre following challenge, compared to a maximum increase of 128-fold in the non-immunised animals. Given that the challenge dose of live organisms would be expected to induce a strong anamnestic response in animals to which it is administered, the data obtained for the Group A animals which had previously received three doses of killed vaccine, are inconsistent with survival and proliferation of the hurstbridge in the vaccinated organisms *in vivo*. In contrast, four of the six Group B animals clearly exhibited signs of infection as determined by MAT.

These data demonstrates that vaccination of the piglets of Group A has inhibited the survival and proliferation of serovar hurstbridge or serogroup Hurstbridge *in vivo*.

TABLE 15

Serum MAT titres for serovar hurstbridge following challenge of vaccinated and control piglets with serovar hurstbridge or serogroup Hurstbridge

	MAT Titres of Serum for serovar hurstbridge				
Animal *	**Day 0**	**Day 1**	**Day 2**	**Day 4**	**Day 10**
Group A:					
Pig 2	128	512	512	512	512
Pig 3	64	128	128	128	128
Pig 4	256	1024	1024	1024	2048
Pig 5	128	128	128	128	512
Pig 6	128	256	256	512	256
Group B:					
Pig 7	0	0	0	0	0
Pig 8	0	0	0	32	2048
Pig 9	0	0	0	0	2048
Pig 10	0	0	0	0	trace
Pig 11	0	0	0	0	2048
Pig 12	0	0	0	0	512

* Pig No. 1 died prior to challenge.

TABLE 16

Increase in MAT titre in response to challenge with serovar hurstbridge or serogroup Hurstbridge in vaccinated and control piglets

Animal *	Day of Challenge	At Slaughter	Increase in Titre*
Group A:			
Pig 2	128	512	4-fold
Pig 3	64	128	2-fold
Pig 4	256	2048	8-fold
Pig 5	126	512	4-fold
Pig 6	128	256	2-fold
Group B:			
Pig 7	0	0	0
Pig 8	0	2048	128-fold
Pig 9	0	2048	128-fold
Pig 10	0	trace	0
Pig 11	0	2048	128-fold
Pig 12	0	512	32-fold

* Pig No. 1 died prior to challenge.

EXAMPLE 14

Production of a rabbit antiserum against serovar hurstbridge or serogroup Hurstbridge isolated from Herd B

Isolate 1 (Table 3) was grown in culture to about 10^8 organisms/ml in Korthof's (protein-free) medium. The culture was heated at 56°C for 30 minutes to kill the leptospires and emulsified with an equal volume of Montanide ISA 50 adjuvant. A rabbit was immunised weekly for six weeks with 2ml of adjuvanted leptospire, each dose being distributed over ten subcutaneous sites. Blood was obtained from the ear two weeks after the last dose.

EQUIVALENTS

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

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SEQUENCE LISTING

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AND Pig Research and Development Corporation

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(iii) NUMBER OF SEQUENCES: 26

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1477 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAATACCTAG CGGCGAACGG GTGAGTAACA CGTGGTAATC TTCCTCCGAG TCTGGGATAA 120

CTTTCCGAAA GGAAAGCTAA TACCGGATAG TCCTGTTGGA TCACAAGATT TGATAGGTAA 180

AGATTTATTG CTTGGAGATG AGCCCGCGGC CGATTAGCTA GTTGGTGAGG TAATGGCTCA 240

CCAAGGCGAC GATCGGTAGC CGGCCTGAGA GGGTGTCCGG CCACAATGGA ACTGAGACAC 300

GGTCCATACT CCTACGGGAG GCAGCAGTTA AGAATCTTGC TCAATGGGGG AAACCCTGAA 360

GCAGCGACGC CGCGTGAACG AAGAAGGTCT TCGGATTGTA AAGTTCATTA GGCAGGAAAA 420

ATAAGCAGCA ATGTGATGAT GGTACCTGCC TAAAGCACCG GCTAACTACG TGCCAGCAGC 480

CGCGGTAATA CGTATGGTGC AAGCGTTGTT CGGAATCATT GGGCGTAAAG GGTGCGTAGG 540

CGGATTTGTA AGTCAGGTGT GAAAACTGCG GGCTCAACCC GTGGCCTGCA CTTGAAACTA 600

CAAGTCTGGA GTTTGGGAGA GGCAAGTGGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAT 660

ATCTGGAGGA ACACCAGTGG CGAAGGCGAC TTGCTGGCTC AAAACTGACG CTGAGGCACG 720

AAAGCGTGGG TAGTAAACGG GATTAGATAC CCCGGTAATC CACGCCCTAA ACGTTGTCTA 780

CCAGTTGTTG GGGGTTTTAA CCCTCAGTAA CGAACCTAAC GGATTAAGTA GACCGCCTGG 840

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GCATGTGGTT TAATTCGATG ATACCCCAAA AACCTCACCT GGGCTTGACA TGGATCTGAA 960

TCATGTAGAG ATATATGAGC CTTCGGGCAG ATTCACAGGT GCTGCATGGT TGTCGTCAGC 1020

TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCCCTATC GTATGTTGCT 1080

ACCTTAAGTT GGGCACTGGT ACGAAACTGC CGGTGACAAA CCGGAGGAAG GCGGGGATGA 1140

CGTCAAATCC TCATGGCCTT TATGTCCAGG GCCACACACG TGCTACAATG GCCGATACAG 1200

AGGGTCGCCA ACTCGCAAGA GGGAGCTAAT CTCTAAAAGT CGGTCCCAGT TCGGATTGGG 1260

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TGAATACGTT CCCGGACCTT GTACACACCG CCCGTCACAC CACCTGAGTG GGGAGCACCC 1380

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GAAGTCGTAA CAAGGTACCG TAAATCGATT CCTGCAG 1477

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTTGGA 7

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGTTGGANNN NNNNNTTTGA TA 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:9:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCGAGTCTGG GATAACTTTC CGAAAGGGGA GCTAATACTG GATGGTCCCG AGAGGTCATA 60

TGATTTTTCG GGTAAAGATT TATTGCTCGG AGCTGAGCCC GCGCCCGATT AGCTAGTTGG 120

TGAGGTAATG GCTCACCAAG GCGACGATCG GTAGCCGGCC TGAGAGGGTG TTCGGCCACA 180

ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 200 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCGAGTCTGG GATAACTTTC CGAAAGGGGA GCTAATACTG GATAGTCCCG ATAGATCATA 60

GGATGTATCG GGTAAAGATT CATTGCTCGG AGATGAGCCC GCGCCCGATT AGCTAGTTGG 120

TGAGGTAAAG GCTCACCAAG GCGACGATCG GTAACCGGCC TGAGAGGGTG TTCGGCCACA 180

ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 200 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGAGTCTGG GATAACTTTC CGAAAGGGGA GCTAATACTG GATAGTCCCG AGAGGTCATA 60

GGATTTTTCG GGTAAAGATT TATTGCTCGG AGATGAGCCC GCGCCCGATT AGCTAGTTGG 120

TGAGGTAATG GCTCACCAAG GCGACGATCG GTAGCCGGCC TGAGAGGGTG TTCGGCCACA 180

ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 200 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGAGTCTGG GATAACTTTC CGAAAGGGAA GCTAATACTG GATAGTCCCG AGAGATCATA 60

AGATTTTTCG GGTAAAGATT CATTGCTCGG AGATGAGCCC GCGTCCGATT AGCTAGTTGG 120

TGAGGTAATG GCTCACCAAG GCGACGATCG GTAGCCGGCC TGAGAGGGTG TTCGGCCACA 180

ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 199 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGAGTCTGG GATAACTTTC CGAAAGGGAA GCTAATACTG GATGGTCCCG AGAGATCATA 60

AGATTTTTCG GGTAAAGATT TATTGCTCGG AGATGAGCCC GCGTCCGATT AGCTAGTTGG 120

TGAGGTAAAG GCTCACCAAG GCGACGATCG GTAGCCGGCC TGAGAGGGTG TTCGGCCACA 180

ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 200 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTGAGTCTGG GATAACTTTC CGAAAGGGAA GCTAATACTG GATGGTCCCG AGAGATCATA 60

AGATTTTTCG GGTAAAGATT TATTGCTCGG AGATGAGCCC GCGTCCGATT AGCTAGTTGG 120

TGAGGTAAAG GCTCACCAAG GCGACGATCG GTAGCCGGCC TGAGAGGGTG TTCGGCCACA 180

ATGGAACTGA GACACGGTCC 200

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATGGATCCA GAGTTTGATC MTGGCTCAG 29

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTGCCAGCMG CCGCGG 16

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAACTYAAAK GAATTGACGG 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGCGCGTCT TAAACATG 18

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAAGTCAAGC GGAGTAGCAA 20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACGGGCGGTG TGTRC 15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGGTTGCGCT CGTTG 15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GWATTACCGC GGCKGCTG 18

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ACCATCATCA CATYGCTGC 19

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTCCCCCCAT TGAGCAAGAT T 21

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTATTTTTCC CTGCTTACTG AAC 23